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Production and Physicochemical Properties of Water-Insoluble Glucan from *Streptococcus mutans*

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A method for the production of water-insoluble glucan was developed by utilizing the extracellular glucosyltransferase present in the culture fluid of *Streptococcus mutans*, and some physicochemical properties of the glucan powder were investigated.

Streptococcus mutans was cultured in brain heart infusion (BHI) medium. The growth phase shifted to the logarithmic phase and the stationary phase at 6 and 14 h after the inoculation of the preculture into BHI medium, respectively. The doubling time was about 180 min.

Water-insoluble glucan was produced by incubation of the cell-free supernatant of the culture fluid with sucrose added as a substrate. The amount of water-insoluble glucan produced was affected by the concentration of substrate, temperature and pH; the optimum values of these factors were 15% (w/w), 37 °C and 7.5, respectively. By gas-liquid chromatographic analysis of the methylated water-insoluble glucan, the glucan chain was found to consist of 60% of α-1,3- and 20% of α-1,6-D-glucosidic bonds. The molar percent of each type of linkage was not affected by the pH of the reaction mixture but was affected by the incubation temperature. Among 8 strains examined, OMZ-176 and 6715 showed the best production of water-insoluble glucan. No peak was observed in the powder X-ray diffraction pattern of the freeze-dried glucan, suggesting that it is an amorphous powder. The hygroscopicity of the glucan powder was similar to that of corn starch. A rheological study showed that a dispersion of the glucan had non-Newtonian and shear-thinning behavior.

Keywords——production method; water-insoluble glucan; *Streptococcus mutans*; physicochemical property; extracellular glucosyltransferase

It has been known for more than twenty years that streptococci have cariogenic action. These cariogenic streptococci are generally called *Streptococcus mutans*. These microorganisms produce water-soluble or insoluble polysaccharides such as glucan and fructan from sucrose by the action of extracellular enzymes.[1,2] Recently water-insoluble glucan has been shown to play an important role in the formation of dental plaque, and many studies on the chemical properties[3,4] and crystal structure[5] of glucan have been reported. There have also been many reports concerning the characteristics of glucosyltransferases which produce water-insoluble glucan from sucrose as a substrate.[6-8] However, there are only a few reports concerning the utilization of this glucan.

Starch and cellulose are well known polysaccharides obtained from plants, and are widely used, intact or as their derivatives, in many fields. On the other hand, dextran is a polysaccharide obtained by using microorganisms. Moreover, it has been confirmed that some polysaccharides produced by microorganisms have unique characteristics different from those of the synthetic polymers. Some of those polysaccharides, *e.g.*, pullulan[9] and curdlan,[10]

which have anomalous flow characteristics, have been put to practical use.

As a part of our investigations concerning the pharmaceutical usefulness of water-insoluble glucan, we have reported that glucan is useful as a vehicle for directly compressed tablets[11] and sustained-release tablets.[12] However, there is no report describing a practical production method. Therefore, in this study, the effects of concentration of substrate, pH and strain of *Streptococcus mutans* on the production of water-insoluble glucan were investigated. Moreover, some physicochemical properties of water-insoluble glucan are presented.

Experimental

Bacterial Strains——*Streptococcus mutans* strains OMZ 176, OMZ 175, OMZ 65, 6715, B 13, MT-615R, EN 19-1 and E 49 were used in this study. All strains were obtained from the Department of Microbiology, Dental School, Osaka University, Osaka, Japan.

Cultural Conditions——The organism was cultured under aerobic conditions in batch culture at 37 °C for 18 h using Brain Heart Infusion Broth (Difco Laboratories, Detroit, Mich.). Preculture was carried out in the same medium for about 24 h. Growth was determined by measuring the absorbance of the culture at 570 nm with a spectrophotometer (UV-240, Shimadzu Corp., Kyoto, Japan).

Production and Fractionation of Glucan——Glucan was produced and fractionated by means of the procedures shown in Chart 1. The culture was harvested in the stationary phase, *i.e.*, 18 h after inoculation, and the cell-free supernatant was obtained by centrifugation at 3000 × *g* for 10 min at 4 °C (KR-2000T, Kubota Medical Appliance Supply Corp., Tokyo, Japan). Unless otherwise stated, the supernatant fraction was incubated at 37 °C for 4—7 d with 15% sucrose and 0.04% sodium azide. The water-insoluble glucan was isolated by centrifugation, washing by distilled water several times and freeze-dried. The water-soluble glucan in the supernatant fraction was fractionated by precipitation with a final concentration of 80% ethanol. The ethanol-insoluble fraction was isolated by centrifugation, washed with 80% ethanol and freeze-dried.

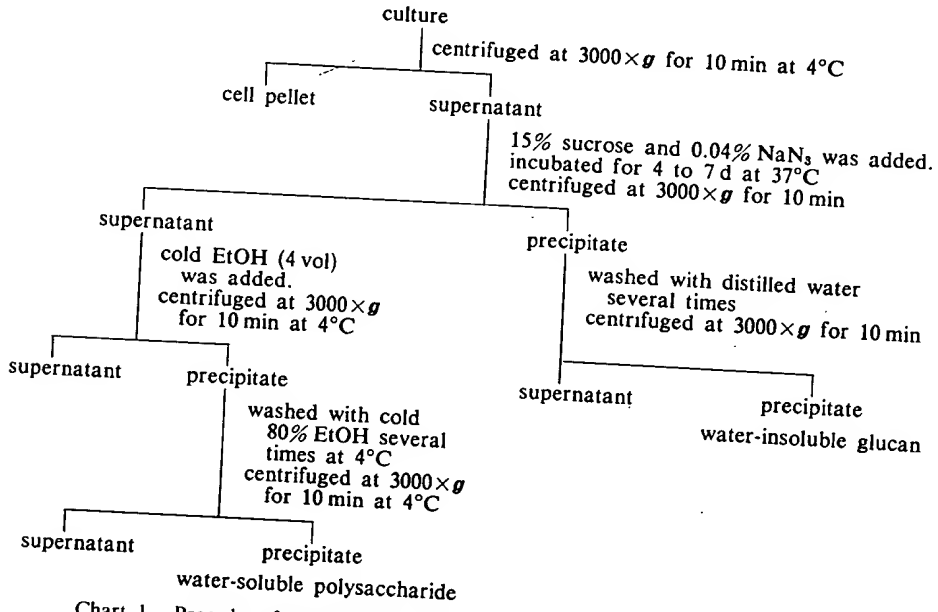

Chart 1. Procedure for Production and Fractionation of Polysaccharides from *S. mutans*

Determination of Glucan and Individual Saccharides in the Reaction Mixture——Water-soluble and insoluble glucan isolated were measured by the phenol sulfuric acid method.[13] Sucrose, glucose and fructose in the reaction mixture were measured by gas-liquid chromatography (GLC) using sorbitol as an internal standard. Cold ethanol (4 volumes) was added to the reaction mixture at 4 °C and the precipitate was removed. The supernatant was diluted with 9 volumes of water and a 1 ml aliquot was evaporated *in vacuo* in a rotary evaporator at 30 °C. The dried sample was treated with 0.5 ml of trimethylsilylating reagent (pyridine: hexamethyldisilazane: trimethylchlorosilane =

10:5:1) for 30 min at room temperature and analyzed by GLC. The GLC apparatus and operating conditions were as follows: instrument, model GC-7A (Shimadzu Corp., Kyoto, Japan); column, 5% silicone GE SE 30, Chromosorb W (AW-DMCS), 3.2 mm × 1.0 m; column temperature, a linear gradient of 170—250 °C at 8 °C/min from 16 min after injection; detector, FID; detector and injection temperatures, 270 °C; carrier gas, N_2, 50 ml/min; H_2 and air pressure, 0.5 kg/cm^2.

Methylation Analysis——Ten milligrams of the glucan dried *in vacuo* over phosphorus pentoxide was suspended in 4 ml of dimethyl sulfoxide at 60 °C in a nitrogen atmosphere, then methylated by the method of Hakomori[14] with 0.5 ml of fresh methylsulfinyl carbanion. The reaction mixture was allowed to stand for 5 h at room temperature, then 1 ml of methyl iodide was added under cooling in an ice bath. The reaction mixture was dialyzed against water overnight at room temperature and the methylated glucan was extracted into chloroform. The extract was evaporated *in vacuo* using a rotary evaporator. The methylated glucan was hydrolyzed with 0.2 ml of 72% sulfuric acid for 1 h at 0 to 5 °C, then for 6 h at 100 °C after dilution with 1.2 ml of water. The hydrolyzate was neutralized with barium carbonate. The methylated sugar components were reduced with 20 mg of sodium borohydride, converted into alditol acetates by treatment with 0.2 ml of pyridine and 0.2 ml of acetic anhydride for 2 h at 100 °C, and dried *in vacuo* using a rotary evaporator. The acetylated glucan was dissolved in chloroform and an aliquot was analyzed by GLC. The apparatus and operating conditions of GLC were as follows: instrument, model GC-7A (Shimadzu Corp., Kyoto, Japan); column, 3% ECNSS-M, Gaschrom Q, 3.2 mm × 2.1 m; column temperature, 190 °C; detector, FID; detector and injection temperatures, 300 °C; carrier gas, N_2, 60 ml/min; H_2 and air pressure, 0.5 kg/cm^2.

Infrared (IR) Spectroscopy——Measurement was carried out by the KBr disc method using an infrared spectrophotometer (model 260-10, Hitachi, Tokyo, Japan).

Powder X-Ray Diffractometry——Powder X-ray diffractometry was carried out using a diffractometer (Geigerflex model D-2, Rigaku Denki, Tokyo, Japan) with Ni-filtered, Cu-K_α radiation.

Study on Flow Characteristics of Glucan Dispersion——Glucan gel (6% (w/w)) was prepared by mixing the glucan and water in a mortar with a pestle, and allowed to stand overnight at 4 °C. Assessment of the flow characteristics of the glucan dispersion was carried out at 20 °C using a rotational viscometer, Haake Rotovisko RV12 (Haake Mess-Technik GmbH) equipped with an M 500 measuring head and MV II sensor system.

Results and Discussion

The production of water-insoluble glucan (WIG) has been observed in many strains of *Streptococcus mutans* (*S. mutans*). In this study we investigated the yield and the nature of WIG produced by several strains of *S. mutans* from the viewpoint of potential pharmaceutical application. The yields of WIG and the water-soluble polysaccharide (WSP) obtained from 8 strains of *S. mutans* are shown in Table I. The yields of polysaccharides varied from strain to strain. Among 8 strains, OMZ 176 and 6715 produced most WIG. In addition, WIG obtained

Table I. Polysaccharide Production by Various Strains of *S. mutans*

Strain	Yield of WIG (g/l)	Yield of WSP (g/l)
OMZ 176	4.0±0.6	13.9±1.1
OMZ 175	0.7±0.2	17.0±2.0
OMZ 65	2.2±0.3	10.0±0.9
6715	4.0±1.0	7.4±1.5
B-13	1.6±0.6	9.7±0.6
MT-615R	0.6±0.5	13.3±2.4
EN 19-1	0.3±0.2	33.8±5.4
E-49	1.5±0.9	8.9±2.6

Production was carried out for 96 h at 37 °C. Each value represents the mean±S.D. of ten and three determinations for OMZ-176 and other strains, respectively.

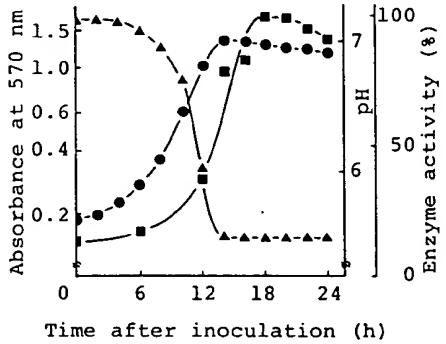

Fig. 1. Changes of Enzyme Activity and pH during Growth

●, absorbance at 570 nm; ■, enzyme activity in the culture fluid; ▲, pH of the culture fluid. The degrees of relative activity are expressed as a percentage of the activity of culture fluid obtained 18 h after inoculation. Each point represents the mean of three determinations.

from OMZ 176 according to the procedure shown in Chart 1 was pulverized most easily, and gave a white powder on freeze-drying. We have previously reported the pharmaceutical availability of WIG from OMZ 176,[11,12] so, OMZ 176 was selected as the preferred strain for all subsequent experiments in this investigation.

Changes of pH and the Enzyme Activity of the Culture Fluid during Growth

Figure 1 illustrates the changes of pH of the culture fluid and the activity of the extracellular glucosyltransferase (GTase) in the culture fluid during growth of the organism. The growth phase shifted to the logarithmic phase and to the stationary phase at 6 and 14 h after the inoculation of the preculture, respectively. The doubling time at the log phase was about 180 min. The initial pH of the culture fluid was 7.2. The pH of the culture fluid decreased with growth and remained at 5.5 after the temperature of growth. On the other hand, the activity of the extracellular GTase in the culture fluid increased with growth after a lag time of about 3 to 6 h. The maximum activity of GTase was observed at 4 h after the termination of growth, *i.e.*, 18 h after the start of the culture. Therefore, in subsequent experiments, the cell-free culture fluid containing GTase was harvested, in principle, 18 h after the start of the culture and utilized as the crude enzyme solution.

Production of the WIG

Time Course of WIG Production——The time course of the production of WIG is shown in Fig. 2. The production of WIG proceeded in proportion to incubation time for about 96 h. However, the rate of production slowed down suddenly at 96 h after the start of the incubation. The WIG preponderantly adhered to the glass surfaces of the vessel in an agglomerated state, and hardly any was observed suspended in the reaction mixture. Dispersion of the adhered WIG by agitation was not easy.

Chemical Changes in the Reaction Mixture during WIG Production——Changes of the concentrations of sucrose, glucose and fructose in the reaction mixture during the production of WIG are shown in Fig. 3. The consumption of the substrate, *i.e.*, sucrose, corresponded quite well to the total production of WIG, WSP, glucose and fructose. A moderate amount of glucose that corresponded to 25% of the sucrose consumed was observed. Gibbons reported

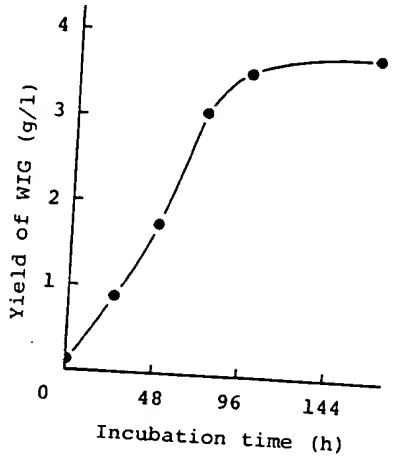

Fig. 2. Time Course of WIG Production

The reaction mixture used was obtained 18 h after the inoculation of the preculture. The pH of the reaction mixture was not regulated. The incubation temperature was 37 °C and the concentration of sucrose was 15%. Each point represents the mean of three determinations.

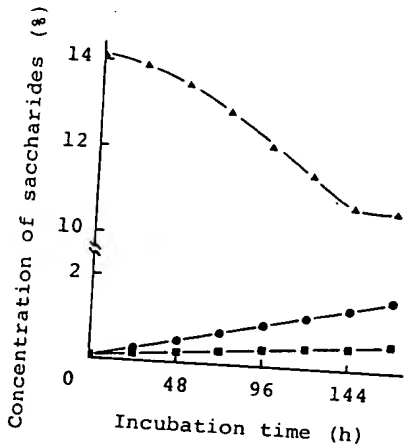

Fig. 3. Chemical Changes in the Reaction Mixture during Polysaccharide Production

▲, sucrose; ■, glucose; ●, fructose. The conditions of production were the same as in Fig. 2. Each point represents the mean of three determinations.

that a cell-associated invertase-like enzyme, which appeared to be similar to invertase on the basis of its catalytic action, its molecular size, and its heat sensitivity, was present in cells of *S. mutans* strains GS 5 and 6715.[15] The same invertase-like enzyme might exist in the culture fluid. On the other hand, glucanase might also be present in the culture fluid. Therefore, the production of glucose might have been due to the action of an invertase-like enzyme and/or glucanase. On the other hand, the production of fructose should be mainly due to the action of the GTase, since the amount of fructose produced was much larger than that of glucose. In addition, it was suggested that the production of water-soluble fructan was small since the amount of fructose produced was about 85% of that calculated from the amount of sucrose consumed.

Effect of Initial Substrate Concentration on WIG Production——The effect of initial substrate concentration on WIG production is shown in Fig. 4. When the cell-free culture fluid or the crude enzyme solution, *e.g.*, a phosphate buffer in which 50% ammonium sulfate-saturated fraction of culture fluid was dissolved,[3] was used as the reaction mixture for WIG or WSP production, the concentration of sucrose applied was reported to be 10%. On the other hand, when the WIG and WSP production was carried out during culture, the concentration of sucrose applied was reported to be 5%.[16] However, we found that the amount of WIG produced was largest when the initial concentration of sucrose was 15%.

Effect of pH on WIG Production——The effects of pH of the reaction mixture on WIG production and on the structure, *i.e.*, molar ratios of linkage types, of WIG produced are shown in Fig. 5 and summarized in Table II, respectively. The amount of WIG produced was the largest when the pH of the reaction mixture was 7.5. The amount of WIG produced at pH 7.5 was about 1.7 times that produced when the pH was not regulated, *i.e.*, pH 5.5. On the other hand, the molar ratios of linkage types of WIG produced at each pH were essentially the same. In addition, the ratios were comparable to those reported for WIG produced by strain OMZ 176.[3,17,18]

GTase has been reported to consist of several components.[6-8] Guggenheim and Newbrun reported that the optimum pH of those components fell in the range of pH 5.0 to

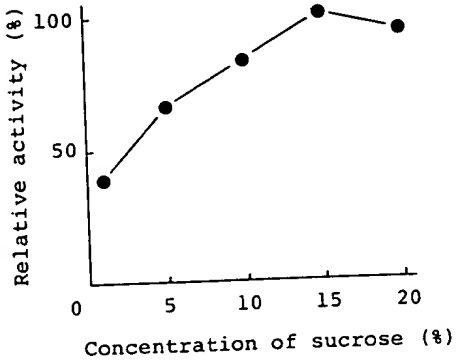

Fig. 4. Effect of Initial Substrate Concentration on WIG Production

The reaction mixtures containing sucrose at various concentrations were incubated at 37 °C for 168 h. The degrees of relative activity are expressed as a percentage of the activity at the concentration of 15% sucrose. Each point represents the mean of three determinations.

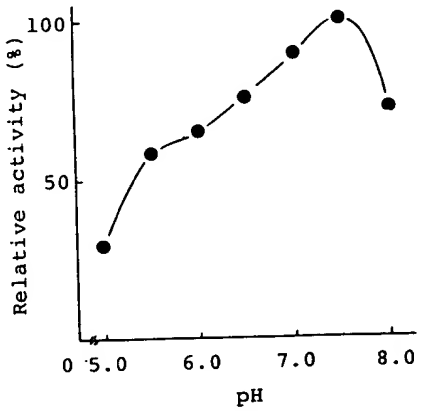

Fig. 5. Effect of pH of the Reaction Mixture on WIG Production

The pH of each reaction mixture was adjusted with McIlvaine's buffer and confirmed not to have changed after WIG production by using a pH meter. Each reaction mixture was incubated for 96 h at 37 °C. The degrees of relative activity are expressed as a percentage of the activity at pH 7.5. Each point represents the mean of three determinations.

Table II. Effect of pH of the Reaction Mixture on WIG Structure

pH	Methyl *O*-methyl-D-glucopyranoside			
	2,3,4,6-Tetra-	2,4,6-Tri-	2,3,4-Tri-	2,4-Di-
5	11±0.6	62±0.6	17±0.6	10±0.6
6	10±0.6	63±2.3	16±2.1	11±1.2
7	13±2.5	57±2.1	19±1.2	11±1.5
8	9±1.2	62±1.5	19±2.0	10±1.0

Each value represents the mean molar percent±S.D. of three determinations. Each WIG was produced at 37 °C.

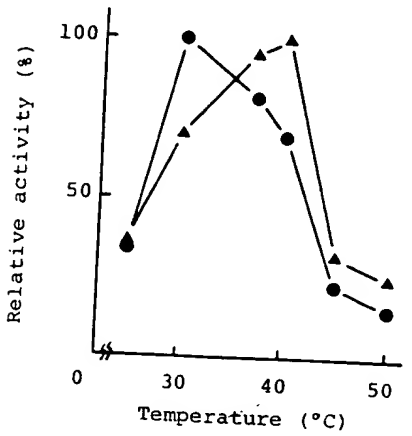

Fig. 6. Effect of Incubation Temperature on WIG Production

Each reaction mixture was incubated for 96 h at 25, 30, 37, 40, 45 and 50 °C. The pH's of the reaction mixtures were 5.5 (▲) and 7.5 (●). The degrees of relative activity are expressed as a percentage of the activity at 40 °C (pH 5.5) or 30 °C (pH 7.5). Each point represents the mean of three determinations.

7.0, yet each showed a distinct optimum and the appearance of WIG produced by each component was different.[6] Therefore, when the production of WIG was carried out by using a mixture of several components, such as cell-free culture fluid used in this experiment, the structure of WIG produced at each pH was expected to be different. However, the methylation analysis in this experiment showed that the structures of WIG produced at different pH values in the range 5.0 to 8.0 were the same, contrary to expectation. Moreover, the adherence of the WIG to the glass surfaces of the vessel was similar at all pH's.

Effect of Incubation Temperature on WIG Production——In Fig. 6, the effect of incubation temperature on WIG production is shown. When the pH was adjusted to 7.5, the amount of WIG produced was the largest at 30 °C. On the other hand, when the pH was not regulated, *i.e.*, pH 5.5, the amount of WIG produced was the largest at 40 °C. Thus, the effect of incubation temperature on the structure of WIG was investigated. The results at pH 7.5 are summarized in Table III; it was found that the ratios of the methyl *O*-methyl-D-glucopyranosides of WIG produced at 25, 30, 37, 40 °C were the same. However, those produced at 45 and 50 °C were very different from those produced at 25 to 40 °C. The WIG produced at 25 to 40 °C contained about 20% of 2,3,4-tri-*O*-methyl-D-glucopyranoside, *i.e.*, α-1,6 linkages, but those produced at 45 and 50 °C contained only 6 to 7% of α-1,6 linkages. Therefore, the activity and/or the stability of the enzyme component which took part in the synthesis of α-1,6 linkages might be low at above 45 °C. Moreover, it was observed that the WIG produced at 45 to 50 °C showed scarcely any adhesive property. Ebisu and Misaki and Koga and Inoue have reported that the adhesive property was attributable to α-1,6 linked chains.[4,19] Therefore, the results obtained in this experiment are consistent with theirs.

Table III. Effect of Incubation Temperature on WIG Structure

Temperature (°C)	Methyl *O*-methyl-D-glucopyranosides			
	2,3,4,6-Tetra-	2,4,6-Tri-	2,3,4-Tri-	2,4-Di-
25	11±1.5	57±2.6	21±2.1	11±1.7
30	12±1.5	57±0.6	20±2.1	11±0.6
37	15±1.5	54±1.2	20±0.6	11±0.6
40	10±1.2	57±1.5	23±0	10±1.0
45	8±1.0	83±1.0	6±1.2	3±0.6
50	5±2.1	85±1.2	7±1.0	3±1.0

Each value represents the mean molar percent ± S.D. of three determinations. Each WIG was produced at pH 7.5.

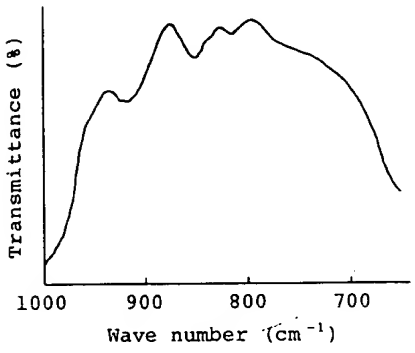

Fig. 7. IR Spectrum of WIG

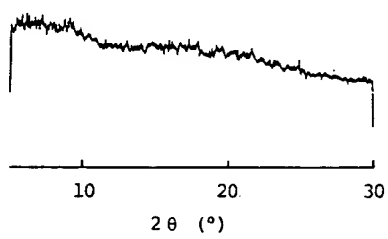

Fig. 8. Powder X-Ray Diffraction Pattern of WIG

In this experiment, the conditions of WIG production were investigated in order to improve tha yield of WIG, but the results are not yet adequate from the viewpoint of industrial production. Other culture conditions need to be investigated in order to further improve the yield of WIG. It was reported that the growth of several strains of *S. mutans* was faster chemically defined medium than in organic medium.[20] Accordingly, we investigated the utility of a chemically defined medium, which was a modification of the PD medium described by Ikeda *et al.*,[21] and was partially defined. Though the yield was improved somewhat, the WIG had a higher content, *i.e.*, about 50%, of α-1,6 linkage and showed a more adhesive character. WIG of this type might be worthy of further investigation. On the other hand Schachtele *et al.*[22] and Robyt and Corrigan[23] have reported that the addition of an exogenous primer such as dextran stimulated the activity of GTase, and Wittenberger *et al.*[24] and Umesaki *et al.*[25] have reported that the addition of Tween 80 stimulated the activity and/or the secretion of GTase. In addition, an increased yield of WIG by control of the salt concentration or the conditions of culture has been reported by Mukasa *et al.*[26] and Kenny and Cole,[27] respectively. Therefore, further examination of the conditions of the culture should lead to further improvement of the yield of WIG.

Physicochemical Properties of WIG

(IR) Spectrum——The IR spectrum of WIG over the range of 650—1000 cm^{-1} is shown in Fig. 7. The spectrum, with absorption bands assigned to α-1,3 linkages (815 and 850 cm^{-1}) and α-1,6 linkages (915 cm^{-1}), resembled that of WIG produced by *S. mutans* Ingbritt A or α-

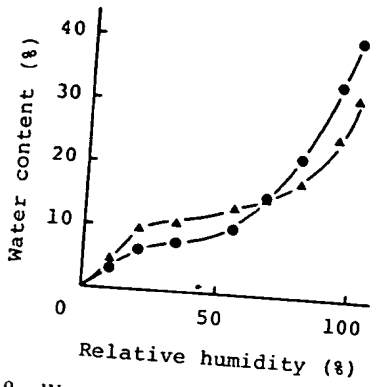

Fig. 9. Water Contents of WIG at Various Levels of Relative Humidity at 20 °C

●, water-insoluble glucan; ▲, corn-starch. Relative humidity was controlled by the use of various saturated salt solutions. Each point represents the mean of three determinations.

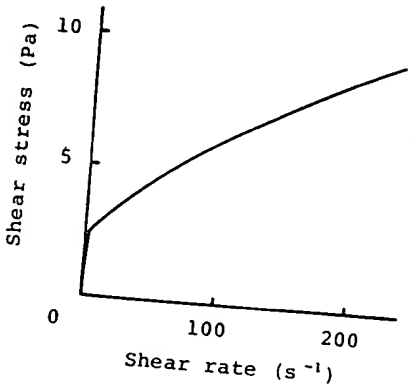

Fig. 10. Flow Curve of WIG Dispersion

The concentration of WIG was 6% and the temperature was 20 °C. The flow curve showed good reproducibility.

1,3 linkage-rich glucan extracted from *Polyporus betulinus*.[3] This result also shows this WIG to be exclusively α-1,3 linked.

Crystallinity——The powder X-ray diffraction pattern of WIG is shown in Fig. 8. WIG had no crystallinity, since no diffraction peak was ovserved in the pattern. Therefore, as reported for other polymers such as polyvinylpyrrolidone,[28] coprecipitates of poorly water-soluble drugs with WIG may be useful to improve the solubility of the drugs. A study on this subject is under consideration.

Hygroscopicity——The relationship between water content and relative humidity is shown in Fig. 9. For comparison, the relationship for corn starch is also shown in the same figure. The relationship for WIG showed a sigmoid-type pattern characteristic of water-insoluble polymers. The hygroscopicity of WIG was less than that of corn starch at under 70% relative humidity.

Flow Characteristic——The flow curve of WIG dispersion is shown in Fig. 10. The curve showed non-Newtonian and shear-thinning behavior. In addition, a yield point was observed. It has been suggested that a pseudoplastic vehicle is better as a suspending agent than a Newtonian vehicle.[29] Therefore, the application of WIG dispersion as a suspending agent may be useful. A rheological study of WIG is under consideration and will be reported elsewhere.

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